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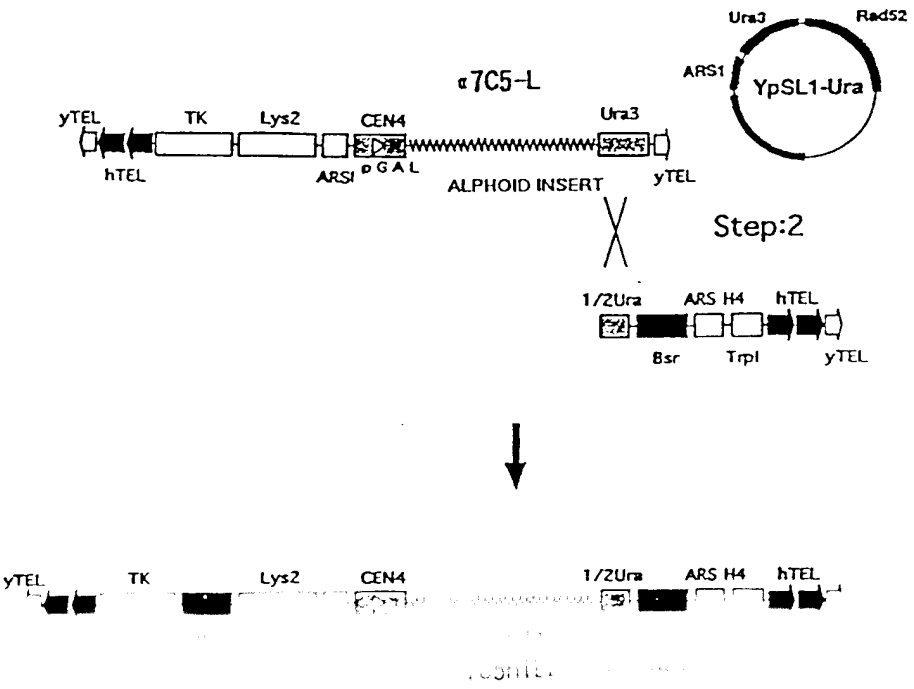

(57) Abstract

A mammalian chromosome is produced according to the method comprising the steps of: introducing a DNA construct comprising a mammalian telomere and a centromere into a mammalian cell, wherein the centromere has a DNA sequence containing some copies of CENP-B box sequence consisting of: 5'-NTTCGNNNNANNCGGGN-3', wherein N is any one of A, T, C and G.

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segments. A new way to generate transgenic mice will be provided by invention of MACs, if their stability during meiosis is established in mammalian development. However, the construction of a MAC has not yet achieved due to the technical difficulties (Willard, Proc. Natl. Acad. Sci. 93, 6874-6850,1996).

Yeast artificial chromosomes (YACs) has been constructed (Burke et al. , Science, 236, 806-812,1987) with three essential DNA elements from the budding yeast, Succharomyces serevisiae; namely, an origin of replication or autonomously replicating sequence (ORI or ARS) required for initiation of DNA replication, telomere sequences (TEL) required to stabilize and facilitate complete replication of chromosomal ends, and a centromere (CEN) required for faithful segrigation of sister chromatids after replication. Since then, YACs became a major tool for cloning of large gene segments of complex genomes. In analogy to YACs, MACs are believed to be constructable with the three essential elements derived from mammalian genomes. Among the three, telomeres have been isolated from mammalian chromosomes and used for the mammalianchromosome manipulation (Brown et al., Hum. Mol. Genet., 27-1237,1994; Farr et al., EMBO J., 14, 5444-5454,1995), but centromeres and origins of replication of mammalian chromosomes were found to be difficult to isolate because of unavailability of activity assays.

The present investigators have analyzed specific structure of mammalian centromere locus in order to reach information on the essential functional structure of mammalian centromeres. They have found that centromere

antibodies (Moroi et al., Proc. Natl. Acad. Sci. USA, 77,1627-1631,1980) at centromeres of various mammalian chromosomes, specifically

he alphoid The present investigation relates to tDNA region with CENP-B box. It is an object of the investigation to provide artificial chromosomes derived from the above region that can be kept stably in extrachromosomal region of mammalial cells, especially in human cells, and be safely transmitted to cells of succeeding generations. The present investigation includes development of methods to construct, modify and stably maintain the precursors of such artificial chromosomes in yeast cells as YACs, which have potential ability to form mammalian artificial chromosomes when introduced into mammalian cells.

[Disclosure of the Invention]

This invention has been achieved by finding a method of constructing an yeast artificial chromosome construct comprising a DNA sequence including CENP-B box sequences from the alphoid DNA region of human chromosome 21 that can interact with CENP-B or the centromere protein B, and a segment of human telomere sequence by the use of homologous recombination in yeast cell, and finding further that when the construct is introduced into a human cell, the construct is replicated autonomously in a human cell and is stably maintained in lineage.

This invention provides a DNA construct comprising a mammalian telomere and a centromere, wherein the centromere has a DNA sequence containing some copies of CENP-B box sequence consisting of : 5'-NTTCGNNNNANNCGGGN-3', wherein N is any one of A,T,C and G.

In a preferred embodiment of this invention, a DNA construct comprising a mammalian telomere and a centromere, wherein the

sequence designated as sequence No. 1.

Preferably, the centromere contains spaced repeats of the CENP-B

deficient host cell with a plasmid for DNA recombination,
(ii) collecting cells carrying the recombinant DNA construct without the plasmid. This invention, allowing a highly efficient intracellular homologous recombination can simplify the conventional in vitro recombination based on the use of restriction enzymes, and thus bring in a new recombination method whereby a given DNA segment can be formed. Preferably, the host cell is a yeast cell. Further, the recombinant DNA construct is of a yeast artificial chromosome. Still further, one of the DNA sequences is of a yeast artificial chromosome. Additionally, one of the DNA sequences is of a yeast artificial chromosome which has a repetitive DNA sequence.

This invention provides a method of establishing a yeast artificial chromosome construct comprising the steps of:
(i) producing a first recombinant yeast artificial chromosome with a mammalian telomere and a centromere in a DNA recombination deficient host cell with a plasmid for DNA recombination, wherein the centromere has a DNA sequence containing some copies of CENP-P box sequence consisting of :
5'-NTTCGNNNNANNCGGGN-3',
wherein N is any one of A,T,C and G.
(ii) selecting cells carrying the first recombinant yeast artificial chromosome without the plasmid.
(iii) Producing a second recombinant yeast artificial chromosome with the telomeres and the centromere from the first recombinant yeast artificial

(iv) selecting cells carrying the second recombinant yeast artificial chromosome without the plasmid.

side of α21-I is composed of diverged alphoid families and may contain other repetitive DNA sequences. CENP-B boxes are distributed regularly in the α21-I locus. On the other hand, the α-21-II locus contains only rare CENP-B boxes.

Fig.2 shows construction of pYAC55pkc used for Alphoid DNA cloning.

Fig.3 shows alphoids DNA inserts which were cloned to YAC55pkc from WAV17 containing the human chromosome 21.

Fig.4(a) shows cloned alphoid DNA inserts by PFGE(stained with EtBr).

Fig.4(b) shows analysis of cloned alphoid DNA inserts by Southern hybridization using α21-I probe.

Fig.4(c) shows analysis of cloned alphoid DNA inserts by Southern hybridization using α21-II probe.

Fig.5 shows alphoid length and detection of other repetitive sequences in alphoid YAC clones by southern and dot hybridization.

Fig.6 shows CENP-B box immunoprecipitation -competition analysis. PCR generated alphoid DNA from alphoid YAC clones were mixed with end labeled CENP-B box DNA and CENP-B , then immunoprecipitation reactions were carried out . The ratio (%) of immunoprecipitated probe was plotted against the amount of the competitor.

Fig.7 schematically shows construction of pMegaΔ using pCGS990.

Fig.8 schematically shows construction of pMega SV-neo using pMegaΔ.

Fig.9 schematically shows construction of pMCU using pJS89.

Fig.10 schematically shows construction of MCU-bsr using pMCU

Fig.12 shows schematically the procedure used for constructing α/C5-left arm.

integration site) of cell lines recloned from 7C5HT1.

Fig.22(a) shows merged image of detection of centromere antigens on minichromosomes and chromosomes. Metaphase chromosomes from cell lines 7C5HT2 was analyzed simultaneously by FISH with α21-I probe (green signals in panel) and YAC arm probe (red signals) .

Fig.22(b) shows merged image of detection of centromere antigens on minichromosomes and chromosomes. Metaphase chromosomes from cell lines 7C5HT2 was analyzed simultaneously by FISH with YAC arm probe (red signals) and by indirect immunofluorescence with anti CENP-B antibody (green signals in panel) .

Fig.22(c) shows merged image of detection of centromere antigens on minichromosomes and chromosomes. Metaphase chromosomes from cell lines 7C5HT2 was analyzed simultaneously by FISH with YAC arm probe (red signals) and by indirect immunofluorescence with anti CENP-C antibody (green signals) .

Fig.23(a) shows merged image of detection of centromere antigens on minichromosomes and chromosomes. Metaphase chromosomes from cell lines 7C5HT1-2 was analyzed simultaneously by FISH with α21-I probe (green signals in panel) and YAC arm probe (red signals) .

Fig.23(b) shows merged image of detection of centromere antigens on minichromosomes and chromosomes. Metaphase chromosomes from cell lines 7C5HT1-2 was analyzed simultaneously by FISH with YAC arm probe (red signals) and by indirect immunofluorescence with anti CENP-B antibody (green signals in panel) .

minichromosomes and chromosomes. Metaphase chromosomes from cell lines 7C5HT1-2 was analyzed simultaneously by FISH with YAC arm

43, 1993; Yoda et al., Mol. Cell Biol., 16, 5169-5177, 1996). Further, CENP-B box sequence is a 17bp sequence designated as sequence No. 1 . The sequence is derived from human chromosome 21(Ikeno et al , Human Mol. Genet., 3, 1245-1257).

The centromere of this invention has spaced repeats of CENP-B box sequence. The centromere has copies or repeats of CENP-B box sequence in sufficient quantity to provide a centromere property to a DNA construct in a host cell. Preferably, the centromere has a region where CENP-B box sequence is contained at high frequency.

A DNA sequence with regular repeats of the CENP-B box sequence which is located at centromere region of human chromosome contains giant repeats with 11 MER as a unit. Each 11 MER body has a size of approximately 1900bp, and each monomer (approximately 170bp) contains five spaced repeats of CENP-B box sequence (Ikeno et al , Human Mol. Genet., 3, 1245-1257).

One of the 11 MER body on human chromosome 21 has been isolated, sequenced and designated as sequence No. 2 (Ikeno et al , Human Mol. Genet., 3, 1245-1257).

It is to be noted that the centromere has a DNA sequence containing some copies of the sequence designated as sequence No.2 or a sequence derived from the sequence No.2 in which one or more nucleotides are added, deleted and/or replaced.

Further, dimers comprising a part of this 11 MER body or another 11 MER body were isolated, sequenced and designated as sequence No. 3 , No.4, No.5, No.6, and No.7[illegible]

(Cloning of centromere)

whole or a part of 11 MER body, or the whole or a part of 2 MER body is made as a probe and hybridized with colonies or the DNAs extracted from transformed cells. This maneuver enables isolation of the transformed cells with CENP-B box sequences of human chromosome.

The centromere DNA sequences which are hybridized with the probes form a part of the alphoid region of human chromosome 21which is designated by the inventors as α21-I region or other alphoid region of mammalian chromosomes.

The α21-I region essentially consists of repeats of 11 MER body, the length of which is approximately 1.3Mbp. This region can be readily extracted from the genome DNAs of WAV 17, a mouse-human hybrid cell containing only human chromosome 21 as genetic materials of human origin.

Other alphoid region of the human chromosome 21 contains very small number of CENP-B box sequence or no CENP-B box sequence. The DNA sequence of this region can be selected in the similar manner described above: human genomes are treated with appropriate restriction enzymes, desired DNA fragments are collected by PFGE and introduced into YACs, the YACs are introduced into yeast cell strains lacking DNA recombination enzymes, α(Y)a and α(Y)b sequences are used as primers and multiplied by PCR, and the PCR products are hybridized with DNA extracted from the yeast cells so that the DNA sequence of interest is detected and cloned. The DNA sequence thus cloned does not contain above DNA sequence with repeats of CENP-B box sequence or the DNA

The sequence constitutes a part of the alphoid region of human

cell, it is necessary that the host cell is competent for homologous recombination and simultaneously able to maintain the recombinant DNA stably. The latter requires conditions defective in DNA recombination. Thus, the homologous recombination in this case consists of the following processes: to allow a plasmid carrying the gene for DNA recombination maintained temporarily in the DNA recombination deficient host cell (The plasmid expresses the DNA recombination enzyme while it stays in the host cell), thereby producing a recombinant DNA through transient homologous recombination, and then to select cells carrying the recombinant DNA without the plasmid.

(DNA recombination deficient host cell)

DNA recombination deficient host cells of the invention include eukaryotic cells containing mammalian cells and yeast cells, and bacterial cells. Preferably, the cells are yeast cells. Further, the cells lacking one or more DNA recombination enzymes are to be used in this invention as the host cell. The host cell with a plasmid carrying the gene for DNA recombination is a host cell of homologous recombination, and the cell without the plasmid is a host cell of maintaining the recombinant DNA construct, which is a stable provider of the recombinant DNA.

The yeast cells lacking DNA recombination enzyme to be used in this invention include the variant lacking the gene rad 51 or rad 52, or the gene coding for the expression of DNA recombination enzymes, or other variants that lacks the genes responsible for the expression of DNA recombination enzymes. Particularly, Saccharomyces cervisiae EPY305-

[illegible] can be mentioned as a recommended material for this invention

(The plasmid for carrying the gene for DNA recombination)

(DNA sequence for recombination)

One DNA sequence to be introduced into host cells should be partially homologous to one of the other DNA sequences to be introduced. Homologous recombination takes place between the two sequences.

DNA sequence for recombination can be linear and / or circular. In each form, DNA sequences can be recombined homologously.

Each DNA sequence for recombination is designed such that the recombinant DNA formed by homologous recombination in cells are replicated autonomously in the host cells and are maintained stably extrachromaomally. In the case of that cells are yeast, the recombinant DNA should have a telomere sequences, an autonomously replicating sequence(ARS) and a centromere(CEN). Thus, the DNA sequence to produce the recombination DNA should contain these segments, so that after the homologous recombination, the recombinant DNA is produced to function as a yeast artificial chromosome.

For example, in order to produce yeast artificial choromosome from two DNA sequence, yeast artificial chromosome is used as one DNA sequence and the other is produced having telomere sequences at 3'-terminal or 5'-terminal and homologous sequences to the yeast artificial choromosome. Then, 3'-terminal or 5'-terminal of the yeast artificial chromosome replaced with the other DNA sequence.

Further, the 3'-terminal and the 5'-terminal of a yeast artificial chromosome may be replaced with a DNA sequence having telomere sequences at 3'-terminal and a DNA sequence having telomere sequences at

the DNA sequence for recombination has a DNA sequence of a gene of interest. After the homologous recombination, the recombinant DNA comes

Yeast artificial chromosome (YAC) used as a DNA sequence for recombination and YAC constructed through homologous recombination in this invention signify DNA fragment which can satisfy at least following requirements; to replicate autonomously in yeast cells and be maintained in their extra chromosomal space. Accordingly, YAC contains telomeres, an autonomously replicating sequence, a centromere, and a sequence necessary for initiating replication, and has these elements arranged in an effective way. Besides, various other YACs well known among those skilled in the art can be utilized with profits.

(YAC for DNA sequence for recombination)

YAC is a useful tool for DNA recombination. YAC containing a DNA sequence of a gene of interest is maintained stably in a yeast cell. Preferably, YAC is used as a DNA sequence for recombination with a large-sized DNA or a repetitive DNA sequence. YAC should be preferably furnished with a selectable marker which allows selective collection of the transformed yeast cells containing the YAC undergoing homologous recombination.

The YAC vector plasmid particularly useful in this invention is pYAC 55pkc or the derivative of pYAC 55 (provided by Dr. Olson, University of Washington). pYAC 55pkc can be prepared as shown in Fig. 6. pYAC 55 is cleaved at Cla I with Cla I and is allowed to have blunt ends. Then, it is allowed to undergo self- ligation in the presence of T4 DNA polymerase. A DNA sequence having Not I sticky ends, and Pst 1(Bst XI), Kpn I, and Cla I positions is inserted into above plasmid at its Not I positions, to

XI), Kpn I and Cla I positions. This plasmid, after being completely cut with Bam HI, produces the YAC that is used in this invention. Or, this

necessary to select the cells carrying the recombinant DNA without the plasmid. This selection takes place by appropriately combining multiple selection markers: the markers contained in the recombinant DNA, the markers removed by the removal of the plasmid and the markers removed from the DNA sequence by the homologous recombination . In the cells selected through above processes, the recombinant DNA can be maintained stably.

(Modifications added to the DNA construct)

Various modifications can be added to the DNA construct having a minimum necessary length of DNA segments, and the resulting construct can be used as a vector to be applied for mammalian cells and other cells.

For example, such DNA construct is made to contain a certain DNA sequence encoding a marker which allows transformed cells to be selected under a specific condition. The selectable marker includes DNA sequences relating to a certain drug resistance or certain nutritive requirements. When the DNA construct is made to contain a DNA segment with such selectable marker, the transformed cells containing the DNA construct can be selectively collected under conditions that a selectively acting chemical or chemicals are present or absent. Such selectable markers are well known among those skilled in the art, and they can choose appropriate markers according to the DNA constructs to be used, based on knowledge commonly shared.

It should be noted here that because the DNA construct of this invention is grown in mammalian cells, the marker must function in

Further, the DNA construct can contain one or more DNA sequences which allow the DNA construct to replicate autonomously in cells other

telomere and a centromere having a DNA sequence containing some copies of CENP-B box sequences through homologous recombination, a YAC having the DNA sequence with CENP-B box sequences is used as a DNA sequence for recombination. This invention comprises two steps of recombination process.

Another DNA sequence (the first arm) necessary for homologous recombination is allowed to contain at least a DNA sequence which is homologous to a DNA sequence located on one end of above YAC, a mammalian telomere, and one or more DNA sequences that are necessary for proper functioning of YAC on one end of the YAC.

The mammalian telomere here has the same meaning with that defined for a newly invented DNA construct.

Further, the DNA sequences necessary for the proper functioning of YAC on one end of the YAC should contain at least a telomere function in yeast cells. If One or more of a centromere, ARS and ORI, exist on one arm of the YAC which is to be replaced with the first DNA arm, the sequences should be provided in addition to a telomere .

The first arm should be preferably furnished with a selectable marker by which the yeast cells having the first recombinant YAC with this DNA arm inserted can be selectively recovered. Further, the first arm should be preferably furnished with a selectable marker by which the mammalian cell having the recombinant YAC construct with this arm inserted can be selectively recovered.

When such first arm is allowed to undergo homologous recombination

[illegible] comprising the

centromere containing CENP-B box sequences, and a mammalian telomere

When homologous recombination is allowed to take place in the yeast cells thus prepared, a second recombinant YAC is obtained wherein the other end of the first recombinant YAC is replaced with the second arm, a DNA sequence with CENP-B box sequences is present, and mammalian telomeres are present at both ends.

The second recombinant YAC produced as above through homologous recombination is furnished also with DNA sequences necessary for it to function in yeast cells. However, if it is inserted into mammalian cells to be maintained in them, those DNA sequences are not always necessary, and can be eliminated.

Furthermore, the recombinant YACs and recombinant DNA construct thus prepared through homologous recombination can be submitted to the same recombination process repeatedly to produce further altered recombinant YACs and DNA constructs.

(The recombinant DNA construct transfection into mammalian cells)

Purified YAC or DNA may be introduced into mammalian cells by the following several methods known in the art. For example, DNA transfections into mammalian cell using lepofectamine(Gibco. BRL) were carried out basically according to the manufactures instruction. Then, MAC transfection was carried out by microinjection. Other methodologies are also useful in the present invention.

YAC or DNA constructs introduced into the cell are maintained stably without integrating into host chromosomes, autonomously replicated, and transmitted to their progeny cells.

[illegible] functional centromere apparently. As a result, it is possible to construct

transformation proceeds without the position effect by the gene integration into the host chromosomes. Particularly, this would be beneficial when used as a vector in gene therapy in which it is necessary to carry normal genes to affected cells, or genes which control the expression of abnormal genes in such affected cells.

Best Mode for Carrying Out the Invention

Having now fully described the present invention, the same will be more clearly understood by reference to certain specific examples which are included herewith for purposes of illustration only, and not intended to be limiting of the invention, unless specified.

Mammalian Cell lines

WAV17, a mouse-human somatic hybrid cell containing the human chromosome 21 with two or three copies per cell as the only human component, was obtained from Dr.F.Ruddle (Yale University, USA) and HT1080, a human male fibroblast cell was obtained from Dr.D.Broccoli (The Rockefeller University). WAV17 and HT1080 were maintained in DME medium (Nissui,Japan) supplemented with 10% fetal calf serum (Bio Whittaker)at 37°C under 5% CO_2.

Antibodies

Anticentromere antibodies (ACA) -positive serum from a scleroderma CREST patient,K.G., which recognize three major centromere antigens, CENP-A, B and C by immunoblotting analysis with Hela nuclear extract, was obtained from Dr.Y.Muro (Nagoya University,Japan). The polyclonal antibody against the NH_2-terminal region of human CENP-B (BN1) was

[illegible] polyclonal antibodies CGP2 and CRa2 against the COOH-terminal region of human CENP-C were raised by immunizing a guinea pig

Top agar: 1M sorbitol, 0.67% yeast nitrogen base without amino acids, 2% glucose, 17% bacto agar, 1x amino acids

SD: 0.67% yeast nitrogen base without amino acids, 2% glucose, 1x amino acids

10x amino acids: 400mg/l arginine-HCl, 200mg/l histidine-HCl, 600mg/l isoleucine, 600mg/l leucine, 500mg/l lysine-HCl, 200mg/l methionine, 500mg/ l phenylalanine, 500mg/ l tyrosine, 100mg/ l adenine sulfate, 2000mg/ l threonone, 400mg/ l tryptophan, 200mg/ l uracil

100x tryptophan: 4.0g/l tryptophan

100x uracil: 2.0g/l uracil

100x lysine: 5.0g/l lysine

Solution I :SCE (for agarose plug) supplemented with 2mg/ml zymolyase 100T (Seikagaku Corporation) and 33mM DTT

Solution II :0.45M EDTA pH9.0, 10mM Tris-HCl pH8.0, 50mM DTT

Solution III :0.45M EDTA pH9.0, 10mM Tris-HCl pH8.0, 1% rauroyl-sarcosine, 1mg/ml proteinase K

YAC vectors and plasmids

Construction of YAC cloning vectors

YAC vector and pYAC55 pkc used for Alphoid DNA cloning are derivatives of pYAC55 (gifted by Dr.M.V. Olson, U. Washington). A Cla I site of pYAC55 was disrupted by digestion with Cla I and self-ligation after the blunt ends creation with T4 DNA polymerase. Then, the derivative plasmid was digested with Not I and oligonucleotides containing Pst I(Bst XI), Kpn I, Cla I sites flanked by Not I sites (YL1 and Y L2)

cloning sites (Fig.2).

resuspended and washed with 50ml of 50mM EDTA. After the centrifugation and removal of all the supernatant, the cell pellet (about 330µl) was resuspended with the same volume of Solution I supplemented with 33mM DTT and 4mg/ml zymolyase 100T (Seikagaku Corporation) and incubated at 37°C for 15 min. Then, the cell suspension was mixed thoroughly with the same volume (about 330µl) of 2% LMP agarose (Seaplaque GTG.) in 0.15M EDTA by pipetting with cut off tip. Using a cut off yellow tip 100µl aliquots of the mixture was poured into plug formers kept on ice. The agarose plugs were transferred into Solution II and incubated at 37°C overnight. Then, the buffer was replaced with Solution III and the plugs were incubated at 50° C overnight. The plugs were stored at 4 °C until use.

Pulse-field gel electrophoresis (PFGE)

PFGE was performed using a pulsaphor electrophoresis unit (Pharmacia) in 1%LE agarose (FMC) and 0.5xTBE (50 mM Tris-HCl, 50 mM Boric Acid and 1 mM EDTA) at 10° C . After ethidium bromide staining and photography, gels were treated with 0.25M HCl for 20min and transferred to nylon membranes (Hybond N , Amersham) in 0.4 M NaOH overnight.

Southern hybridization

Hybridization probes were prepared by labeling gel-purified insert fragments of plasmid DNA or PCR ^{32}P-dCTP using the random primer method. Nylon membranes to which DNA was transferred from the gel of PFGE, were prehybridized and hybridized under conditions of 50% salmon sperm DNA and supplemented with or without probe DNA at 42°C. Final washing was performed in 0.1 x SSC and 0.1% SDS at 68° C.

between the motives in the repeats were amplified. α(1)a/b primers were used to amplify alphoid sequence cloned in α7C5 YAC, and α(Y)a/b primers were used to amplify alphoid sequence cloned in αB13 YAC.

primer sequences:

α(1)a 5'ACAGAAGCATTCTCAGAA 3'

α(1)b 5'TTCTGAGAATGCTTCTGT 3'

α(Y)a 5'-AGAAACTTCTTTGTGATG-3'

α(Y)b 5'-CATCACAAAGAAGTTTCT-3'

All PCR reactions were carried out in a 50 ml reaction mixture containing 10 mM Tris-HCL pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM dNTPs and 1 unit of Taq polymerase (Perkin-Elmer) using 1 mM of primers and 1 ng of WAV17 genomic DNA or yeast genomic DNA containing a YAC. 30 reaction cycles were performed, consisting of 30 sec denaturation at 94° C, 90 sec annealing at 55°C or 57°C and 60 sec extension at 73°C. PCR products were precipitated with ethanol to remove primers and used as probes.

Fluorescence in situ hybridization

Metaphase cells of transformants arrested by colcemid or TN16 (Wako Pure chemical) and interphase cells of HT1080 and derivatives were fixed in methanol/acetate (3 : 1) and spread on coverslips with conventional procedure. FISH was carried out according to the method described by Masumoto et al 1989, Exp. Cell Res., 181, 181-196. Biotin-labeled probe was detected with FITC conjugated avidin (1 :250 dilution with 4× SSC, 1% skim milk, Vector) and digoxigenin-labeled

dilution with 4× SSC, 1% skim milk, Boehringer Mannheim)

incubation at 37°C for 1 hr. Chromosomes and nuclei were counterstained

DNA Probes for FISH

Probes were prepared by the random primer method or nick translation using labeled dUTP as a substrate. Gel purified 11 mer body described as sequence NO.2 (11-4 alphoid DNA) (for detection of the α21-I locus and clones,),α(Y)a/b primed PCR products (for detection of α21- II locus and clones), YAC vectors (telomere sequences were removed by Cla I•Kpn I digestion or Cla I - Xho I digestion from the vectors) and a telomere sequence (TTAGGG)n were labeled with biotin-11-dUTP (Enzo Biochem) or digoxigenin-11-dUTP(Boehringer Mannheim). After removal of free nucleotides in the reaction mixture by a Sephadex G-50 spun column, probes were precipitated with ethanol and dissolved in formamide.

EXAMPLE 1

Cloning of alphoid DNA Arrays in the Human Chromosome 21 into YAC

We tried to clone the centromeric alphoid DNA regions into YAC using rad 52⁻ yeast host, EPY305-5b.

One agarose plug (100μl) containing WAV17 genomic DNA was divided into four pieces and digested completely with Bgl I (60U), Bgl II (60U) or Bam HI (72U) at 37°C overnight. The reaction was stopped by adding final 50m M EDTA and solution was replaced with NDS (0.45M EDTA, 1% rauroyl-sarcosine, 10mM Tris- HCl pH7.4) and stored at 4°C. The plugs were equilibrated with 1xTBE for 2hr and loaded on 1% pre-cooled LMT agarose gel. After removing short DNA fragments less than 50 kb by PFGE (90v,30 sec.pulse,for 70 to 90 min.), the agarose plugs

DNA from the human chromosome 21 was concentrated up to 8 to 10 folds as compared with the starting fraction of bulk genomic DNA. The

pelleted at 1500rpm for 5min at room temperature, and washed once with 25ml of water and then washed with 25ml of 1M sorbitol, and spinned again. The cells were resuspended with 20ml of SCE and mixed with 40μl of β-mercaptoethanol. 100 µl of the samples was removed from the suspension and the starting OD800 was determined after diluting with 900 µl of water. The cell suspension was mixed with 100µl of 2mg/ml Zymolyase and incubated at 30°C. The incubation was stopped when the OD800 of the sample decreased to 75-80% of the starting value. Then the spheroplasted cells were pelleted at 950rpm for 5min, resuspended and washed gently with 15ml of STC twice. The cells were pelleted again and resuspended in 1ml of STC. 100µl each of the spheroplasted cells were mixed gently with 10µl of the transforming YAC DNA prepared in Example 1, incubated at room temperature for 10min, then mixed with PEG solution and incubated at room temperature for 10min. The spheroplasts were pelleted at 950rpm for 5min, and the supernatant was removed as much as possible, then resuspended with 200µl of SOS and incubated at 30°C for 30min. The cells were pelleted and resuspended with SD(-ura -trp). The suspension was mixed with 7ml of melted at 50°C TOP agar and poured onto five prewarmed at 42°C SORB plates (-ura -trp). The plates were incubated at 25°C for 5-7 days.

YAC libraries were constructed with genomic DNA of human and mouse somatic cell hybrid (WAV17) containing chromosome 21 as an only human component and about 10000 colonies of the resulting YAC libraries were screened with alphoid DNA probes (an alphoid 11 monomer higher

Hum. Mol. Genet. 3, 1245-1257, 1994). We obtained 4 and 7 stable alphoid YAC clones containing α21-I and α21-II arrays, respectively.

(Thesis, Edinburgh University, U.K.), which contains 0.5 kb of mammalian telomere repeats flanked by 0.3 kb of yeast TG1-3 telomere repeats. An oligonucleotide containing the 18 bp Isce I recognition site (Boehringer) flanked by Bam Hl compatible ends was inserted into a Bam Hl site located at the junction of the 0.6 kb and 0.8 kb elements. Only one Bam Hl site was retained, immediately downstream of the Isce I site. The 0.6 kb element followed by an Isce I site then the 0.8 kb element constitutes the omega cassette.

The left arm replacement vectors are modified versions of pCGS990 described previously (Smith et al 1993; Mammalian Genome, 4, 141-147). pCG990 was a gift from D. T. Moir, Collaborative Research, Inc. A 2.6 kb Sal I-Cla I fragment which contains a neomycin resistance gene derived from pMC1polA (Stratagene) and a copy of the omega cassette was cloned into Sal I-Cla I digested pCGS990, resulting in the construction of pMeganeo (Fig.7). Tetra Hymena telomere sequence and the neomycin gene in pMeganeo were removed by Not I complete digestion and Xhol partial digestion and the plasmid was circularized with ligation after the creation blunt ends with T4 DNA polymerase treatment. Then, oligonucleotides containing Sal I, Cla I and Not I sites flanked by Cla I compatible sites(YL3 and YL4) were inserted into the Cla I site of the derivative plasmid, resulting in the construction of pMegaΔ retaining only one Sal I, Cla I and Not I site (Fig. 8). A 3.6 kb ApaLI - EcoRI fragment containing a neomycin resistance gene derived from pSV2-neo was treated with T4 DNA polymerase and cloned into the partial digested EcoRI site

pMegaSV-Neo (Fig.) pMega2 or pMega3 were used for the replacement of the alphoid YAC left arms by homologous recombination

EXAMPLE 3

RAD52 plasmid mediated transient homologous recombination in rad52⁻ host

YAC strains and homologous recombination procedure

For the centromere functional assay in vivo, the terminals of the linear DNA fragment should be stabilized to avoid integration, degradation from the ends and end replication problems. Therefore we replaced the left and right arms of these two YAC clones into human telomere sequences. YAC can be modified very easily using yeast homologous recombination systems (Pachnis et al 1990, Proc. Natl. Acad. Sci., 87, 5109-5113, Pavan, 1990, Proc. Natl. Acad. Sci., 87, 1300-1304), but in this case, a dilemma is that we used rad52⁻ host to stabilize the repetitive DNA in the YAC . To overcome this problem, we developed a retrofitting method transiently inducible in rad52- host.

The alphoid YAC arms were replaced with the modified YAC vectors by homologous recombination mediated by transient expression of RAD52 gene in rad 52⁻ host. In the first step, α7C5 or αB13 yeast cells were co-transfected with the linealized left arm replacement vector (MegaΔ or MegaSV-Neo respectively) and a RAD52 expressing plasmid containing TRP1 gene (YpSL1) (Fig.11 and 12). Yeast colony showing +LYS, +URA and -trp phenotype indicates that the left arm replacement was successfully carried out (Fig.13). The RAD52 expressing plasmid, YpSL1, contains yeast ARS fragment but no yeast CEN sequence. Therefore, the plasmid rapidly disappeared from the transfected cells in the absence of TRP

colonies showing [illegible] phenotype [illegible]

replaced YAC arms of the clones were certified by PFGE and Southern

containing RAD52 gene (1st:YpSL1 and 2nd:YpSL1-Ura) and 50μg of single stranded salmon sperm carrier DNA, and then mixed with 300 μl of 40% PEG 4000 solution. The yeast and DNA mixture was incubated at 30°C for 30min with agitation and heat shocked at 42°C for 15min. The yeast cells were collected by spinning down for 5 sec. at 7000rpm, resuspended with 1ml YPD and incubated at 30°C with agitation for 2hr. incubation. The yeast cells were collected and washed with the selection medium SD(1st: -lys -ura, 2nd: -trp -lys) and placed on the appropriate selection SORB plate. After 4-5 days incubation at 25°C, colonies were replicated on with or without Trp SORB plate (1st: +/-trp, 2nd: +/-ura) and incubated at 25°C. After 3-4 days incubation, colonies showing appropriate phenotype (1st: +LYS +URA -trp, 2nd: +TRP +LYS -ura) were picked up and were analyzed the insert size and the existence of human telomere by PFGE and Southern Hybridization. In the first homologous recombination step, YAC55 pkc left arm was replaced with linealized Mega Δ or MegaSV-Neo. The frequency of colonies with a -trp/ +LYS +URA phenotype for the 1st recombination were 14-30%. Yeast strains containing a correctly modified left arm YAC were used for the 2nd recombination of the right arm with linealized MCU-Bsr replacement vector. The frequency of colonies with a -ura/ +TRP +LYS phenotype for the 2nd recombination were 23-42%. Yeast strains correctly modified both arms were maintained on -lys -trp plates and used for the materials for YAC DNA purification and YAC DNA transfection into human cells.

The recombinant YAC containing α7C5insert was designated α7C5 h TEL. Saccharomyces cerevisiae containing α7C5 h TEL (Saccaromyces cerevisiae EPY305-5b α7C5 h TEL) was deposited to Agency of Industrial

which YAC DNA was concentrated, equilibrated with 1xTAE supplemented 100mM NaCl and all the fluid was removed. Agarose of the gel was then melted at 68°C for 10min. incubation and digested with 50U agarase (Sigma) per 1ml of gel slice at 42° for 4hr. The resulting YAC DNA solution was dialyzed (concentrate) using Ultra Free C3 (Millipore) or Microcon 100 (Amicon) against the buffer containing (100mM NaCl, 10mM Tris pH7.5, 1mM EDTA), and the concentration and the integrity of the DNA was checked by conventional gel and PFGE. Between 50 to 100ng purified DNA were obtained from this scale of experiments. Then the purified DNA was used as a material for YAC transfection into human culture cells by lipofection and microinjection.

EXAMPLE 5

YAC transfection into human cells by lipofection and by microinjection

Our modified YAC constructs have total 1.1 kb human telomere sequences flanked by 0.3 kb yeast telomere sequences on both chromosome ends. However, to minimize the possibility that introduced YAC DNA is integrated into host chromosome by low telomerase activity of the host cells, we chose human HT1080 cells reported as high Telomere Associated Chromosome Fragmentation (TACF) activity (Barnett et al ., 1993, Nucleic Acids Research, 21, 27-36) as a host. The purified YAC DNAs were introduced into human HT1080 cultured cells using two different methods, lipofection and microinjection into nuclei.

YAC transfection by lipofection

BRL) were carried out basically according to the manufacturer's instruction. Purified YAC DNA solution (30-100 ng DNA in 300μl

microinjection was performed with a condition of P1; 5000 hPa, P2; 30-60 hPa, P2; 30-60 hPa, P3; 15-30 hPa. After the microinjection, the liquid paraffin oil and the mdeium were removed from the dish, and the fresh medium were added, and the cells were incubated at 37°C for 36 hr in 5% CO_2. Blasticidine S selection (4 mg/ml) was started at this point. In several experiments, the injected cells were continued to grow on the etched cover slips, and derived BS resistant colonies were counted per injected cells on the grid and picked up. In the rest experiments, the injected cells were transferred into a 10 cm dish and started the BS selection. A BS resistant colony was obtained per every 100 to 300 YAC DNA injected cells.

EXAMPLE 6

FATE of Alphoid YACs in HT1080 cells

If these MAC candidates retain all the cis elements required for a mammalian chromosome stability for example, telomeres, a functional centromere/kinetochore structure and a replication origin etc , YAC DNA introduced into the human cells may possibly be maintained without integrating into host chromosomes even after BS selections. To examine this possibility, we analyzed the distributions of the YAC DNA in 24, 20 and 5 cell lines of BS resistant colonies obtained from α7C5hTEL , αB13hTEL and the linealized MCU-Bsr vector introduced cells, respectively, by fluorescent in situ hybridization (FISH) (Fig. 18). Though the number of cells containing a minichromosome which is detectable as an extra chromosomal overlapping signals of α21-I probe

such a minichromosome were observed in the most cell lines (11/11 cell lines or 10/11 cell lines) derived from α7C5hTEL YAC transfection

chromosomes, cell by cell , in the same cell line, the most of the integrations of αB13hTEL YAC or the linealized MCU-Bsr vector in the same cell line were derived from a single site. These results indicate that the integrations occurred as multiple events and independently through the establishment of 7C5HT cell lines. However, the integration occurred only once through the establishment of many B13HT or MCUHT cell lines.

All these results clearly indicate that α7C5hTEL YAC retains the elements required for de novo formation of a human minichromosome very efficiently, but, αB13hTEL does not.

EXAMPLE 7

Stability of Artificial Minichromosome without selection

Previous reports indicated that extrachromosomal elements without centromere function rapidly disappeared when the drug for the selection was excluded from the medium (Huxley). The stability of the minichromosome in three 7C5HT cell lines obtained by lipofection (7C5HT1, 2 and 3) and two 7C5HT cell lines obtained by microinjection (7C5HTm1 and 7C5HTm3) were analyzed both by FISH and colony formation efficiency on plate (plating efficiency: number of colonies on +BS selection against that on non-selective media) after 20, 40, 60 days of passage on non-selective media (Fig.20). Plating efficiency data from these 5 cell lines indicated that in all 5 cell lines more than 50% of population kept BS resistancy even after the 60 days of passage on non-selective media, and 97% of 7C5HT1 and 7C5HT3 cells kept BS resistancy after the off selection. Corresponding to these data, the data

and 7C5HT3 cell lines were very stable even after the passage on non-selective media for 60 days, and the minichromosomes in 7C5HT2,

containing the CENP-B boxes in high frequency (α7C5hTEL YAC) could form minichromosomes at high frequency, although an alphoid YAC containing no CENP-B box (αB13hTEL YAC) could not . This result indicates that CENP-B must be an essentially important factor for de novo formation of functional centromere structure. CENP-C has reported to be localized at the inner layer of kinetochore structure and also reported to be localized only at the functional side of the centromeres in dicentric chromosomes (Saitoh et al. 1992, Cell, 70, 115-125, Sullivan, 1995, Hum. Mol. Gent., 4, 2189-2197). In order to clarify the molecular bases required for the functional centromere/kinetochore structure of human chromosomes, we analyzed distributions of these two centromere proteins on more than 20 each of minichromosomes in 7C5HT1, 1-1, 1-2, and 7C5HT2 cell lines derived from α7C5hTEL YAC introduction (Figs.22, 23 and 24) using simultaneous staining with indirect immunofluorescence and FISH . Clear CENP-B and CENP-C double dot signals were observed on all the minichromosomes which were detected simultaneously by the YAC vector probe, indicating that the minichromosomes contained essentially important protein factors as centromere/kinetochore components. We could not obtain any positive signals of CENP-B staining on the integration sites of αB13hTEL YAC in B13HT1 cell line.

Multiple previous reports which introduced the human alphoid DNA into mammalian cultured cells showed that the anaphase lagging chromosomes were observed according to alphoid DNA integration to the host chromosomes and this phenomenon might be one of functions of alphoid

anaphase cells each from

minichromosome is properly aligned at metaphase plate or not, and the

SEQUENCE LISTING

SEQ ID NO:1
SEQUENCE LENGTH :17
SEQUENCE TYPE :nucleic acid
STRANDEDNESS :double
TOPOLOGY :linear
MOLECULE TYPE :Genomic DNA
ORIGINAL SOURCE :Homo sapiens
SEQUENCE DESCRIPTION:SEQ ID NO:1:

NTTCGTTGGA AACGGGA

SEQ ID NO:3
SEQUENCE LENGTH:338
SEQUENCE TYPE :nucleicacid
STRANDEDNESS :double
TOPOLOGY :linear
MOLECULE TYPE :Genomic DNA
ORIGINAL SOURCE:Homo sapiens,Chromosome21,Centromeric region,WAV17 cell
SEQUENCE DESCRIPTION:SEQ ID NO:3:

AATTCGTATA AACACTAGAC AGCAGCATTC CCAGAAATTT CTTTCGGATA TTTCCATTCA
ACTCATAGAG ATGAACATGG CCTTTCATAG AGCAGGTTTG AAACACTCTT TTTGTAGTTT
GTGGAAGTGG ACATTTCGAT CGCCTTGACG CTACGGTGAA AAAGGAAATA TCTTCCCATA
AAAAATAGAC AGAAGCATTC TCAGAAACTT GTTGGTATAT GTGTACTCAA CTAACAGAGT
TGAACTTTGC CATTGATAGA GAGCAGTTTT GAAACACTCT TTTCGTGGAA TCTGCAAGTG
GATATTTGGA TAGCTTGGAG GATTTCGTTG GAAGCGGG

SEQ ID NO:5
SEQUENCE LENGTH:339
SEQUENCE TYPE :nucleic acid
STRANDEDNESS :double
TOPOLOGY :linear
MOLECULE TYPE :Genomic DNA
ORIGINAL SOURCE:Homo sapiens,Chromosome21,Centromeric region,WAV17 cell
SEQUENCE DESCRIPTION:SEQ ID NO:5:

AATTCGTATA AACACTAGAC AGCAGCATTC CCAGAAATTT CTTTCGGATA TTTCCATTCA
ACTCATAGAG ATGAACATGG CCTTTCATAG AGCAGGTTTG AAACACTCTT TTTGTAGTTT
GTGGAAGTGG ACATTTCGAT CGCCTTGACG GCTACGGTGA AAAAGGAAAT ATCTTCCCAT
AAAAAATAGA CAGAAGCATT CTCAGAAACT TGTTGGTGAT ATGTGTCCTC AACTAACAGA
GTTAAACTTT GCCATTGATA GAGAGCAGTT TTGAAACACT CTTTTTTTGG AATCTGCAAG
TGATATTTGA ATAGTTTGGA GGATTTCGTT GGAAGCGGG

SEQ ID NO:7
SEQUENCE LENGTH:338
SEQUENCE TYPE :nucleic acid
STRANDEDNESS :double
TOPOLOGY :linear
MOLECULE TYPE :Genomic DNA
ORIGINAL SOURCE:Homo sapiens,Chromosome21,Centromeric region,WAV17 cell
SEQUENCE DESCRIPTION:SEQ ID NO:7:

AATTCGTATA AAAACTAGAC AGCAGCATTC CCAGAAATTT CTTTCGGATA TTTCCATTCA
ACTCATAGAG ATGAACATGG CCTTTCATAG AGCAGGTTTG AAACACTCTT TTTGTAGTTT
GTGGAAGTGG ACATTTCGAT CGCCTTGACG CCTACGGTGA AAAAGGAAAT ATCTTCCCAT
AAAAATAGAC AGAAGCATTC TCAGAAACTT GTTGGTGATA TGTGTCTCAA CTAACAGAGT
TGAACTTTGC CATTGATAGA GAGCAGTTTT GAAACACTCT TTTTGTGGAA TCTGCAAGTG
GATATTTGGA TAGTTTGGAG GATTTCGTTG GAAGCGGG

a chromosome in a human cell.

10.The DNA construct of Claim 8, which is capable of being maintained as a chromosome in a mouse cell.

11.A host cell transformed with the DNA construct of any one of Claims 1 to 4 .

12. The host cell of Claim 11, which is a human cell.

13.The host cell of Claim 11, which is a yeast cell.

14. The host cell of Claim 11, which is a mouse cell.

15. The DNA construct of Claim 8 , which further comprises a sequence of a gene of interest.

16. The DNA construct of Claim 8, which further comprises a genome DNA sequence containing structural region and its regulatory region.

17.A method of homologous recombination comprising the steps of:

(i) producing a recombinant DNA construct from two or more linear and/or circular DNA sequences partially homologous in a DNA recombination deficient host cell with a plasmid for DNA recombination,

(ii) collecting cells carrying the recombinant DNA construct without the plasmid.

18.The method of homologous recombination of Claim 17, wherein the host cell is a yeast cell.

19. The method of homologous recombination of any one of Claims 17 and 18, wherein the recombinant DNA construct is of a yeast artificial chromosome.

20. The method of homologous recombination of anyone of Claims 17, 18 and 19 wherein one of the DNA sequences is of a yeast artificial

21.The method of homologous recombination of any one of Claims 17, 18

25. A method of producing a mammalian artificial chromosome of Claim 23, wherein the mammalian cell is a mouse cell.

26. A method of producing a mammalian artificial chromosome of Claim 23, wherein the DNA construct is derived from Saccharomyces cerevisiae α7C5hTEL designated as FERM BP- 5625.

27. A mammalian artificial chromosome which is produced according to the method of any one of Claims 23,24, 25 and 26.

28. A method of fragmentation of a chromosome comprising the step of : introducing a DNA construct comprising a mammalian telomere, a centromere and a DNA sequence partialy homologous to the chromosome and a DNA construct comprising a mammlian telomere and a DNA sequence partialy homologous to the chromosome into the mammalian cell, wherein the centromere has a DNA sequence containing some copies of CENP-B box sequence consisting of :

5'-NTTCGNNNNANNCGGGN-3',

wherein N is any one of A,T,C and G.

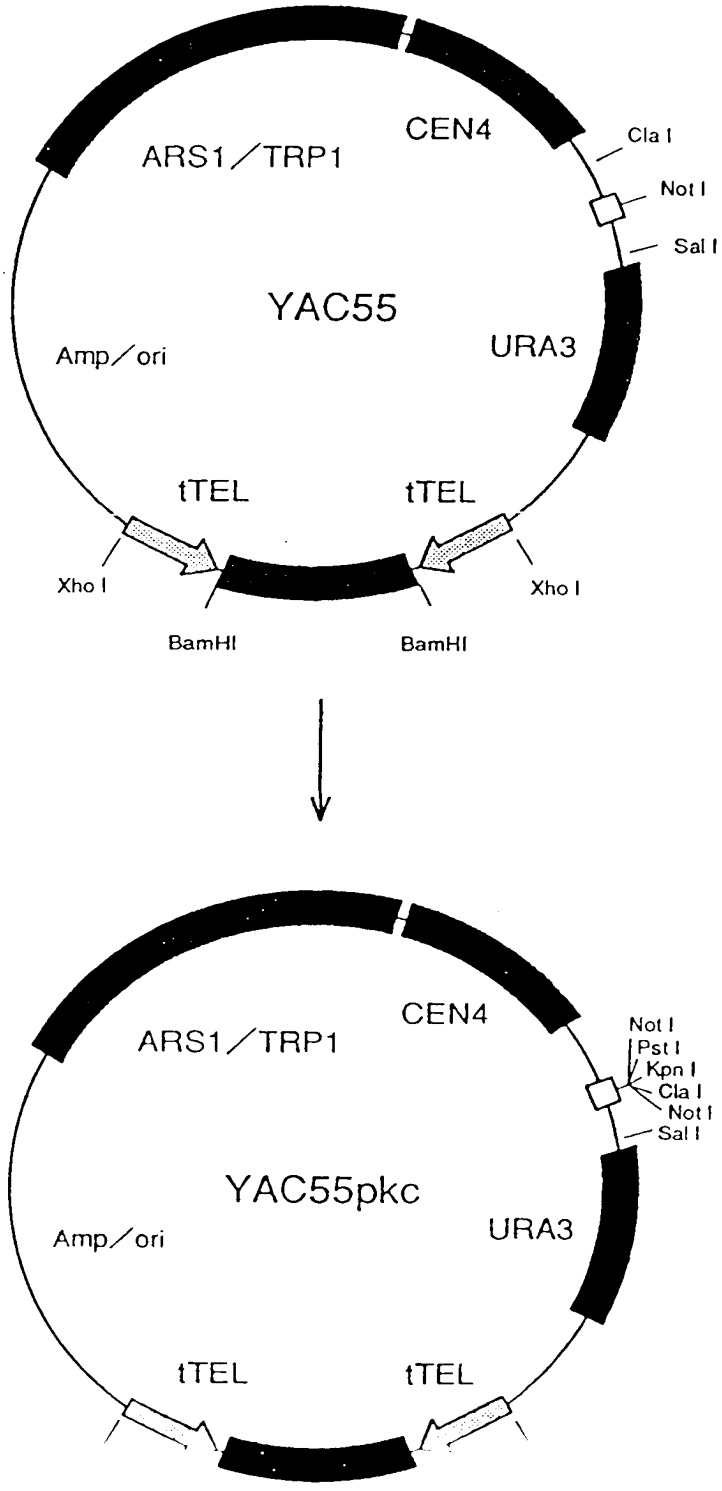
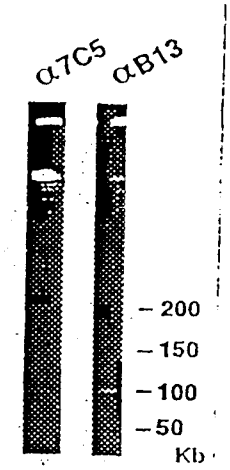

CEN4
ARS1/TRP1
Cla I
Not I
Sal I
YAC55
Amp/ori
URA3
tTEL
tTEL
Xho I
Xho I
BamHI
BamHI
CEN4
ARS1/TRP1
Not I
Pst I
Kpn I
Cla I
Not I
Sal I
YAC55pkc
Amp/ori
URA3
tTEL
tTEL

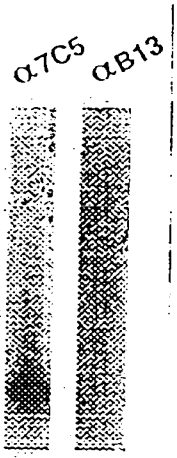
FIG.2

Southern Analysis of Alphoid YACs



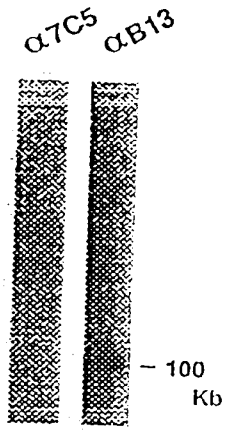
EtBr Stain

FIG.4(a)



Probe: α21-I

FIG.4(b)



α21-II

FIG.4(c)

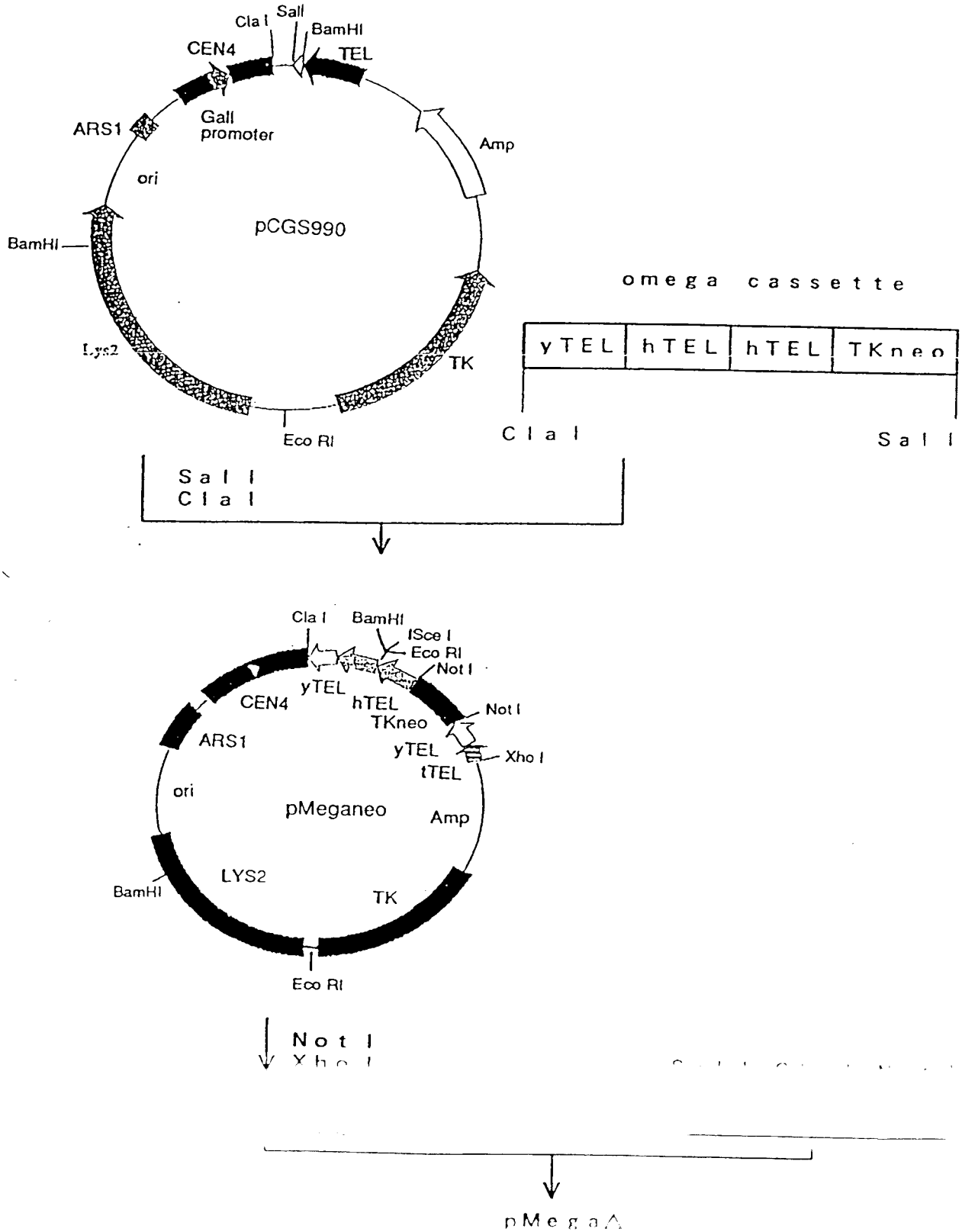

Cla I
Sal I
BamHI
CEN4
TEL
ARS1
Gall promoter
Amp
ori
pCGS990
BamHI
Lys2
TK
Eco RI
omega cassette
yTEL
hTEL
hTEL
TKneo
Cla I
Sal I
Sal I
Cla I
Cla I
BamHI
ISce I
Eco RI
Not I
yTEL
CEN4
hTEL
TKneo
Not I
ARS1
yTEL
Xho I
tTEL
ori
pMeganeo
Amp
LYS2
BamHI
TK
Eco RI
Not I
Xho I
pMegaA

FIG.7

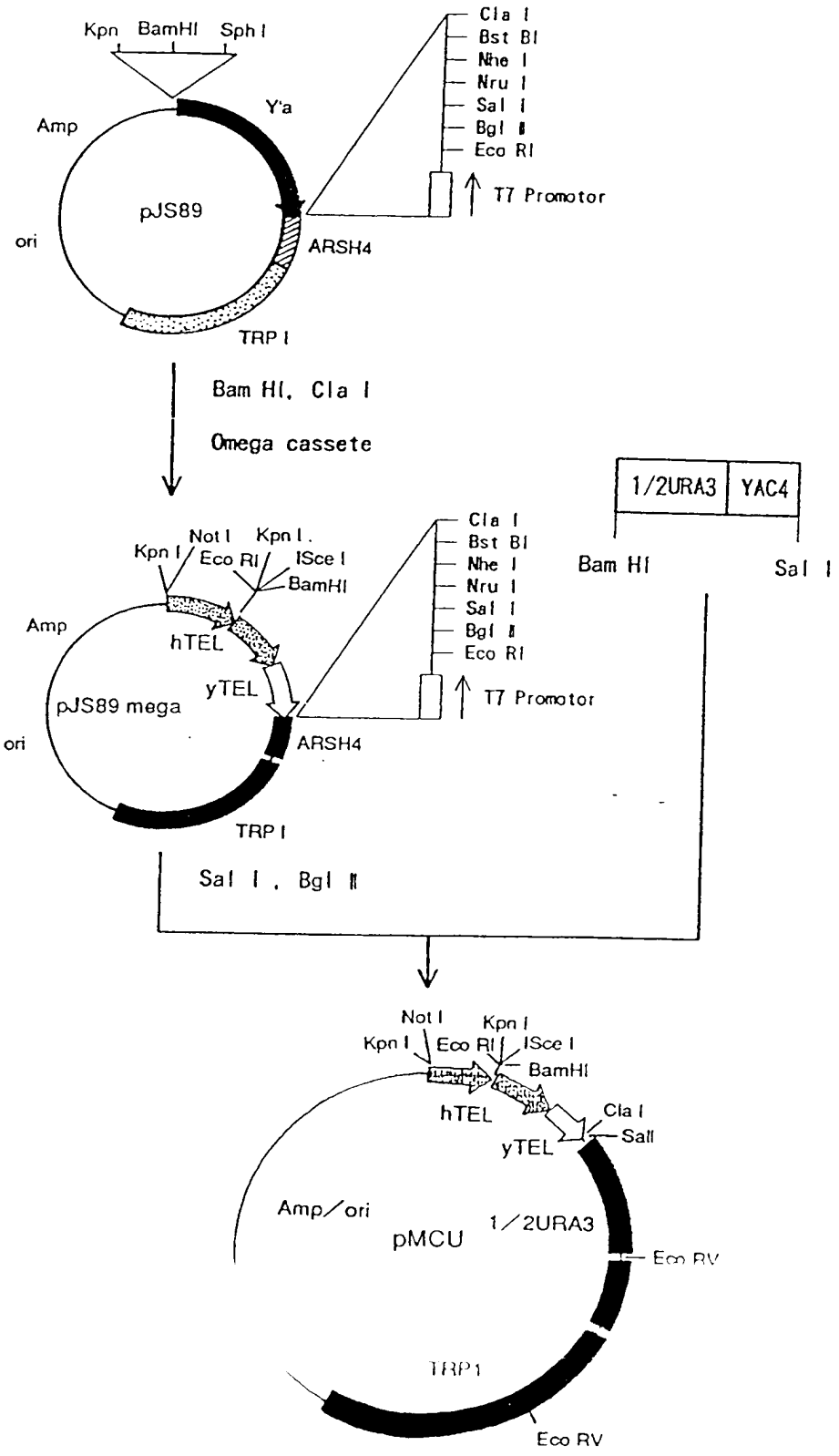

Kpn BamHI Sph I
Y'a
Amp
pJS89
ori
ARSH4
TRP I
Cla I
Bst BI
Nhe I
Nru I
Sal I
Bgl II
Eco RI
T7 Promotor
Bam HI, Cla I
Omega cassete
1/2URA3
YAC4
Bam HI
Sal I
Not I
Kpn I
Kpn I
Eco RI
ISce I
BamHI
Amp
hTEL
yTEL
pJS89 mega
ori
ARSH4
TRP I
Cla I
Bst BI
Nhe I
Nru I
Sal I
Bgl II
Eco RI
T7 Promotor
Sal I , Bgl II
Not I
Kpn I
Kpn I
Eco RI
ISce I
BamHI
hTEL
Cla I
Sall
yTEL
Amp/ori
pMCU
1/2URA3
Eco RV
TRP1
Eco RV

FIG.9

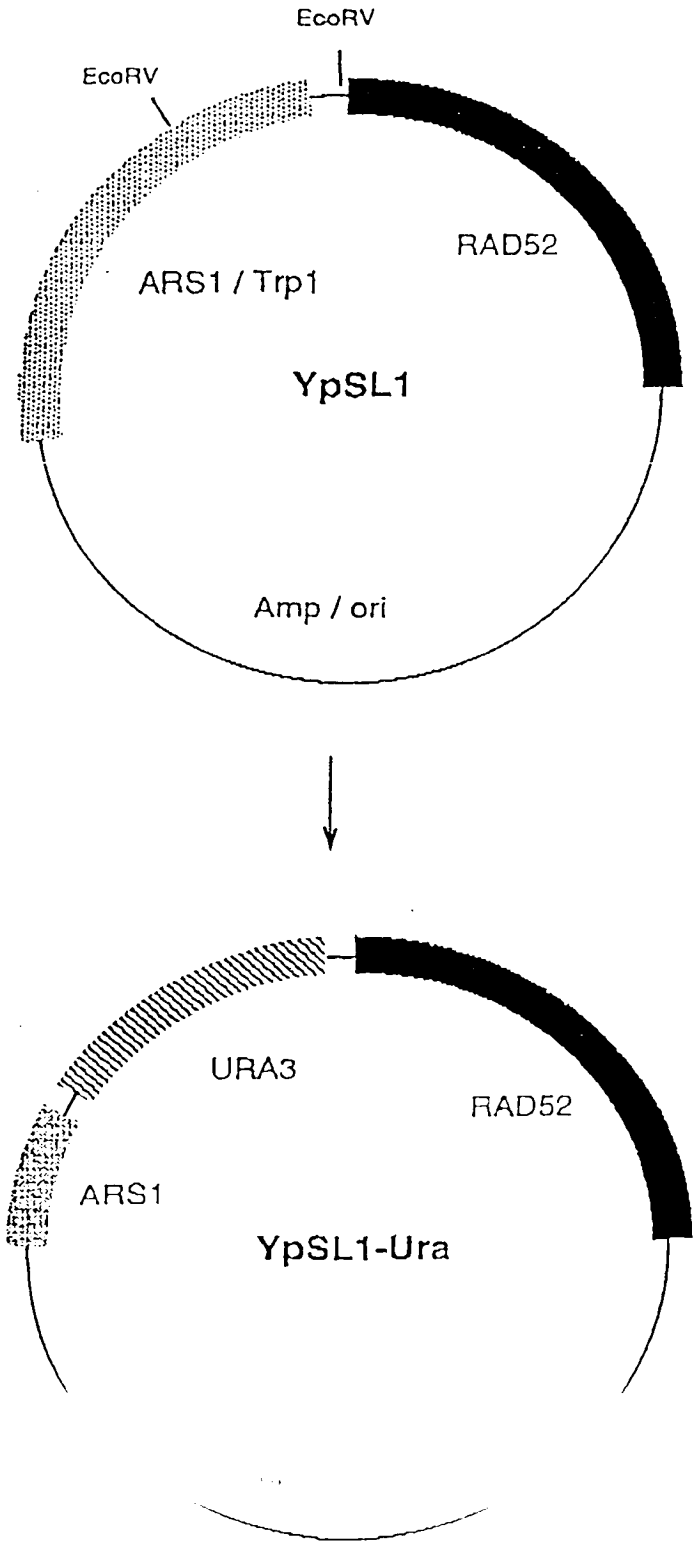

EcoRV
EcoRV
RAD52
ARS1 / Trp1
YpSL1
Amp / ori
URA3
RAD52
ARS1
YpSL1-Ura

FIG.11

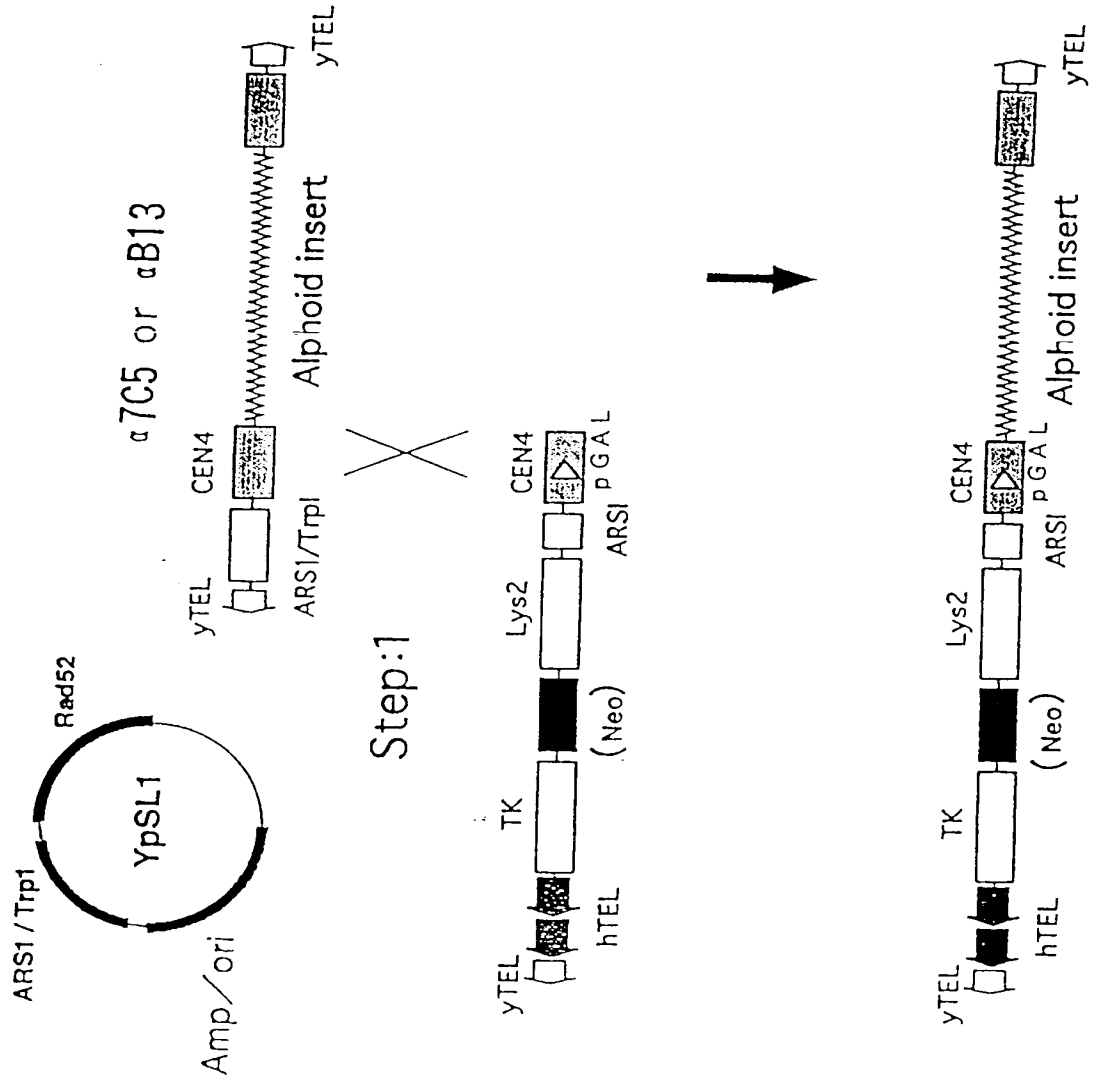

ARS1 / Trp1
YpSL1
Rad52
Amp/ori
αC5 or αB13
yTEL
CEN4
ARS1/Trp1
Alphoid insert
yTEL
Step:1
yTEL
TK
Lys2
CEN4
pGAL
ARSI
hTEL
(Neo)
yTEL
TK
Lys2
CEN4
pGAL
ARSI
hTEL
(Neo)
Alphoid insert
yTEL
α7C5-L

FIG.13

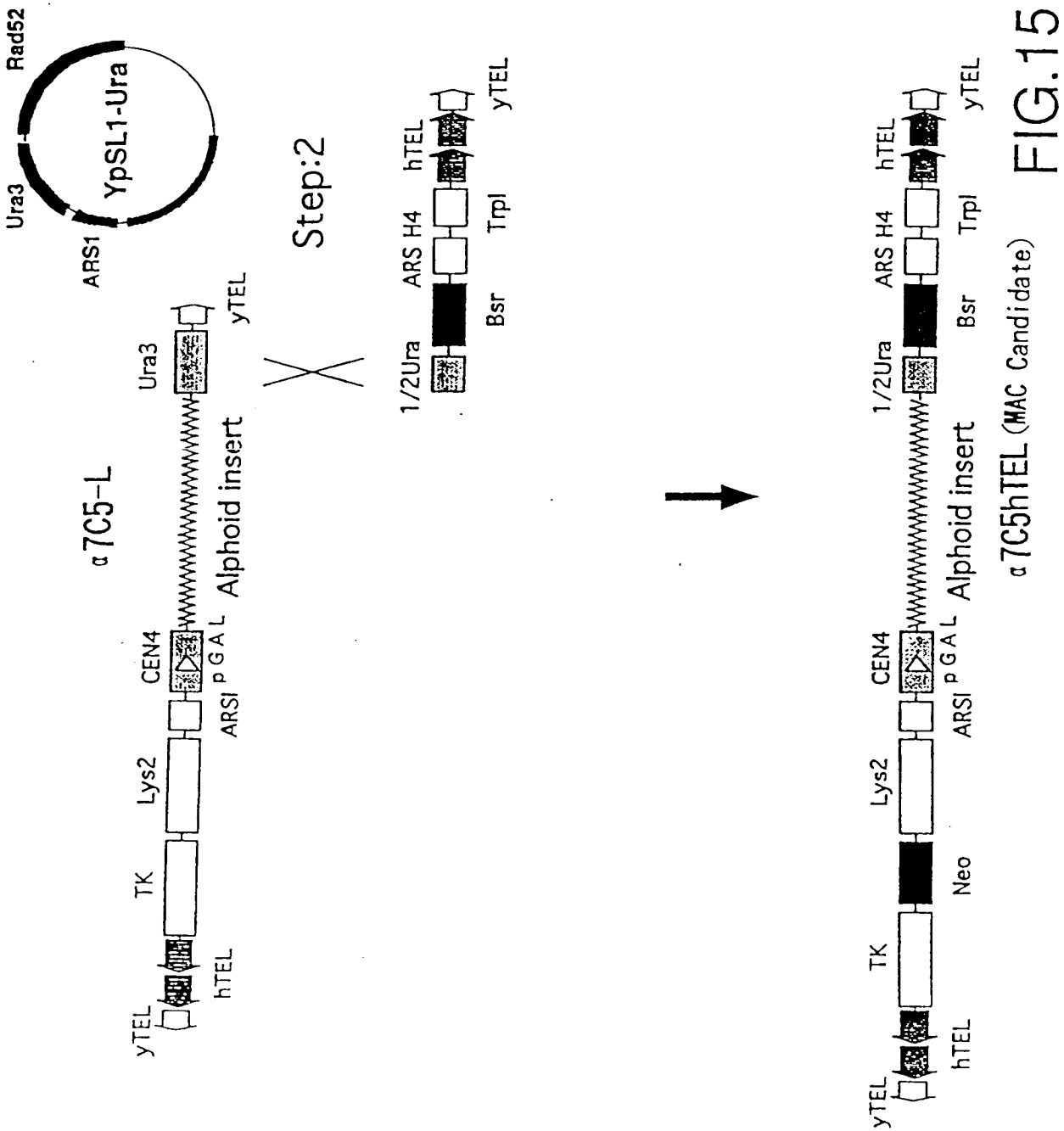

Rad52
Ura3
YpSL1-Ura
ARS1
α7C5-L
Ura3
yTEL
Alphoid insert
CEN4
pGAL
ARSI
Lys2
TK
hTEL
yTEL
Step:2
1/2Ura
ARS H4
hTEL
Bsr
TrpI
yTEL
α7C5hTEL (MAC Candidate)
Neo

FIG.15

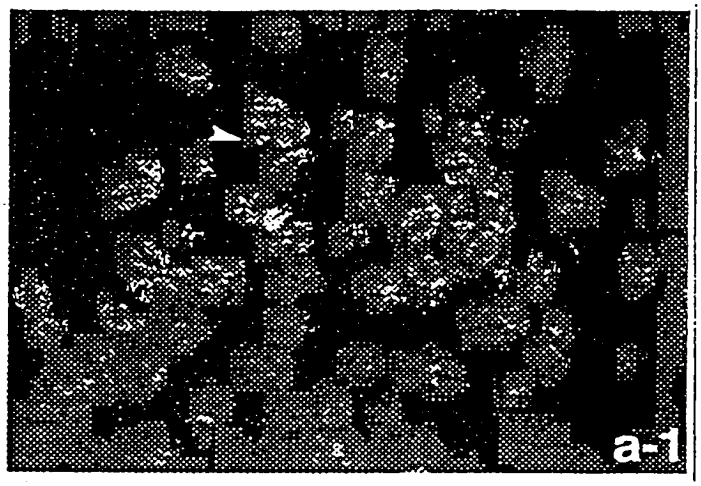


FIG.18(a)

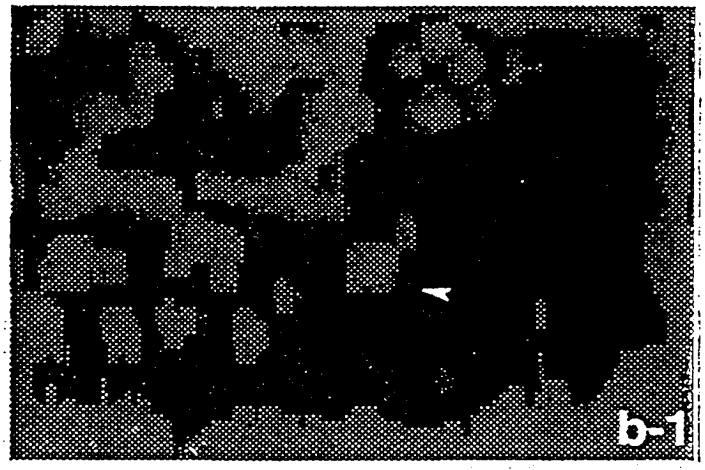


FIG.18(b)

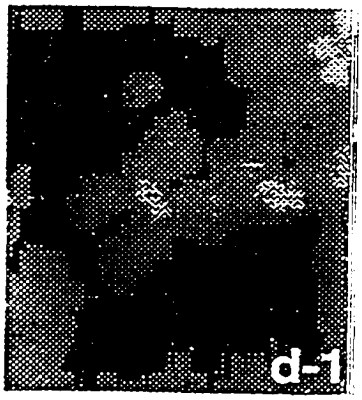


FIG.18(c)

Stability of the alphoid YAC-derived minichromosome as determined by plating efficiency and by FISH analysis

Cell line	Days off selection (generations)	Plating efficiency: Colony No. on +BS selection / that of off selection	FISH analysis: Cell No. showing the signals							
			Total M cells	Minichromosome/cell 1	2	3	Integration site Tel	Cen	Arm	No signal
7C5HT1	0	-	50 (%)	29 (58)	4 (8)	1 (2)	3 (6)	13 (26)	0	0
	20	630 / 627 (100.4%)	-							
	40	634 / 642 (98.7%)	-							
	60	655 / 672 (97.4%)	50	30 (60)	0	0	1 (2)	17 (34)	0	2 (4)
7C5HT2	0	-	50	9 (18)	0	0	14 (28)	0	0	27 (54)
7C5HT3	0	-	50	26 (52)	6 (12)	1 (2)	2 (4)	15 (30)	0	0
	20	610 / 614 (99.3%)	-							
	40	658 / 668 (99.5%)	-							
	60	631 / 652 (96.8%)	50	25 (50)	0	0	2 (4)	17 (34)	0	6 (12)
7C5HTm1	0	-	40	14 (35)	5 (13)	0	1 (3)	0	0	20 (50)
7C5HTm3	0	-	40	2 (5)	0	0	36 (90)	0	0	2 (5)

Day 0 is the time on which each cell line was established and already had passed 30 to 40 days from the YAC DNA transfections.

FIG.20

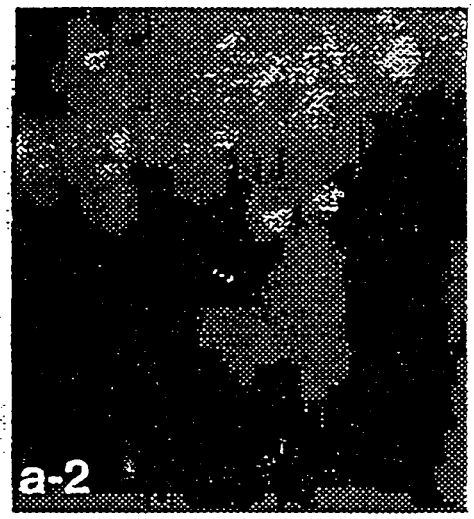


FIG.22(a)

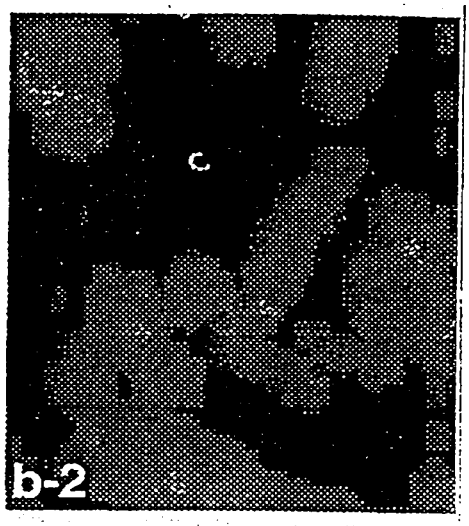


FIG.22(b)

FIG.22(c)

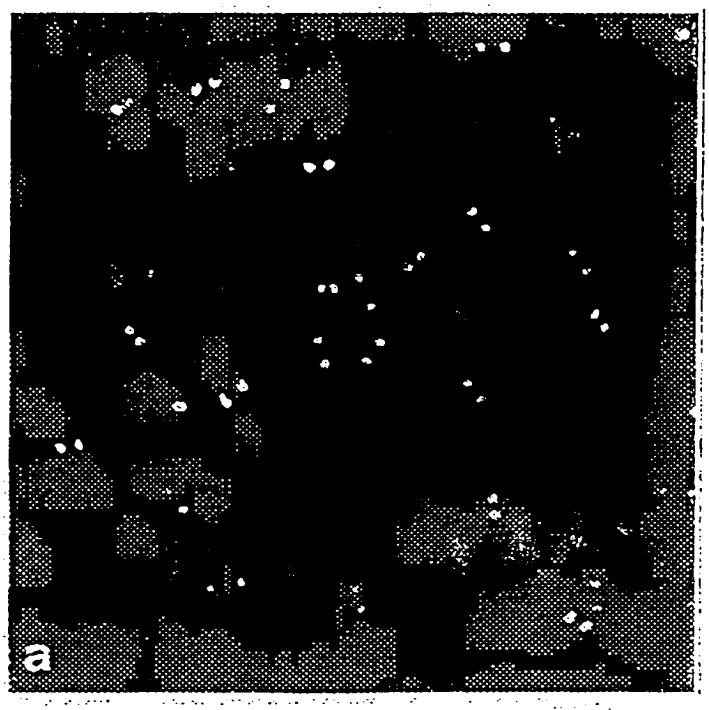


FIG.24

Applicant's or agent's file reference number	T8- -73	International application No.	PCT/JP96/C -381

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 44, line 26

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution
National Institute of Bioscience and Human-Technology
Agency of Industrial Science and Technology

Address of depositary institution *(including postal code and country)*

1-3, Higashi 1-chome, Tsukuba-shi, IBARAKI 305 JAPAN

Date of deposit	Accession Number
14.08.96	FERM BP-5625

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

[ ] This sheet was received with the international application	[X] This sheet was received by the International Bureau on
03.10.96	21 OCT 1996
Authorized officer	Authorized officer
目 磯 睦 子	Masaki Sakai

Form PCT/RO/134 (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 88, 1991, WASHINGTON, DC, US, pages 5744-5748, XP000673332 C. CAMPBELL ET AL.: "Generation of a nested series of interstitial deletions in yeast artificial chromosomes carrying human DNA" see the whole document	17-21
Y	HUMAN MOLECULAR GENETICS, vol. 3, no. 8, 1994, OXFORD, GB, pages 1245-1257, XP000644753 M. IKENO ET AL.: "Distribution of CENP-B boxes reflected in CREST centromere antigenic sites on long-range alpha-satellite DNA arrays of human chromosome 21" cited in the application see materials and methods: page 1256 and Table 1. see the whole document	1-16, 22-28
Y	CURRENT OPINION IN GENETICS AND DEVELOPMENT, vol. 2, 1992, OXFORD, GB, pages 479-486, XP000600761 W.R.A. BROWN: "Mammalian artificial chromosomes" see the whole document	1-16, 22-28
A	BIO/TECHNOLOGY, vol. 12, 1994, NEW YORK, US, pages 586-590, XP000606299 C. HUXLEY ET AL.: "Ordering up big MACs" see the whole document	1-16, 22-28
A	HUMAN MOLECULAR GENETICS, vol. 3, no. 8, 1994, OXFORD, GB, pages 1383-1386, XP000601479 S.S. TAYLOR ET AL.: "Addition of functional human telomeres to YACs" see the whole document	1-16, 22-28
A	WO 95 32297 A (CANCER RESEARCH CAMPAIGN TECHNOLOGY LIMITED) 30 November 1995 see the whole document, specially : see page 1, line 1-37 - page 2, line 1-24 see page 3, line 2-8 see page 4, line 1-14	1-16, 22-28

INTERNATIONAL SEAR[redacted]REPORT

International application No.
PCT/[redacted]06/02381

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see continuation-sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information [redacted] patent family members

Inter [redacted] Application No
PCT/[redacted] 6/02381

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		EP 0593631 A	27-04-94
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